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egg quality;
hatching asynchrony;
maternal effects;
sex ratio.

Abstract

Female birds of several species have control over the production of daughters and sons. However, most studies failed to find a relationship between egg size and sex. This is intriguing as adjustment of egg size would constitute a powerful tool for the female to meet different resource demands of the sexes, particularly in size dimorphic species. Our results show that, within clutches of black-headed gulls (*Larus ridibundus*) the proportion of males was positively associated with egg mass. This applied for all three laying positions, independently of the absolute egg mass. There was a significant relationship between the distribution of the sexes over the laying sequence and the egg mass change. When egg mass decreased over the sequence, first-laid eggs were male biased and last-laid eggs female biased, and vice versa. The potential adaptive value of this allocation strategy is evaluated with regard to male sensitivity to egg quality and competitive differences between the sexes.

Introduction

As reproduction is costly current reproductive events should be traded off against future reproduction, especially in long-lived species (Stearns, 1992). Female birds can reduce their costs of reproduction by laying lighter and fewer eggs. Furthermore, if one sex is more costly to rear and its fitness more heavily dependent on the nutritional circumstances, mothers should adjust the brood sex ratio towards the sex that profits most from given circumstances (Trivers & Willard, 1973). This hypothesis is supported by evidence on biased offspring sex ratios in response to food abundance and female condition in avian species, where one sex is more vulnerable to harsh conditions (e.g. tawny owl *Strix aluco*: Appleby *et al.*, 1997, zebra finch *Taenopygia guttata*: Kilner, 1998). This may relate to sex differences in energetic requirements in the case of species with sexual size dimorphism (Anderson *et al.*, 1993a; Dijkstra *et al.*, 1998; Krijgsveld *et al.*, 1998; Riedstra *et al.*, 1998). The theoretical predictions made by Trivers & Willard (1973) apply across a wide range of species, whenever the fitness of producing sons or daughters varies predictably with the circumstances under which the offspring is produced (Charnov, 1979, 1982). These circumstances may not only be described by female condition or environmental factors, but also by the quality of the oocyte. This has indeed been shown for lesser black-backed gulls *Larus fuscus*, black-headed gulls *Larus ridibundus* and jackdaws *Corvus monedula*, where males were more susceptible to a reduction of size and/or quality of the eggs (Nager *et al.*, 1999; Arnold & Griffiths, 2003, W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished). Thus females may not only numerically produce more offspring of a particular sex, but also skew their distribution of resources between the sexes to maximize inclusive fitness (Charnov, 1982).

Although the ability of mothers to manipulate the sex of the embryo according to her condition and/or food availability has been shown in a number of experimental studies (lesser black-backed gull: Nager *et al.*, 1999, great skua *Catharacta skua*: Kalmbach *et al.*, 2001, zebra finch: Rutkowska & Cichon, 2002), these studies failed to find a direct relationship between egg mass and offspring sex although their experimental food manipulation significantly affected egg mass. The absence of such a relationship is intriguing, as chicks benefit from

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enhanced egg mass (reviewed in Williams, 1994; Christians, 2002), and females may thus have a tool to diminish the higher vulnerability of the larger sex (Nager *et al.*, 1999, W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished). Furthermore, the results of the studies that did find overall sex differences in egg mass have been rather inconsistent, as high egg mass was correlated with the smaller sex (American kestrel *Falco sparverius* Anderson *et al.*, 1997, spotless starling *Sturnus unicolor* Cordero *et al.*, 2001, brown song lark *Cinclorhamphus cruralis* Magrath *et al.*, 2003) or larger sex (house sparrow *Passer domesticus* Cordero *et al.*, 2000, jackdaw Arnold & Griffiths, 2003). The interpretation of these results becomes even more complicated because within and between clutch effects are often insufficiently distinguished in the statistical analyses. This makes it difficult to draw conclusions on both proximate and ultimate aspects of such co-variation.

Females may bias the sex ratio of their eggs according to their rearing capacities (between clutch adjustment), in such a case the absolute egg will be of importance. Thus theoretically, in a between nest comparison light clutches and consequently light eggs, produced by females with a reduced rearing capacity and low body condition, should be biased towards the sex that is less affected by a reduced egg size and quality.

Females may also adjust the sex of each individual egg according to the relative size of the egg in comparison with the other eggs of that particular female, which is independent of the size of eggs laid by other females. Such a sex specific division of her resources would allow the female to interfere with competitive differences between the sexes and to respond to differential nutritional needs of the sexes (within clutch adjustment).

In order to distinguish between these two possibilities, we investigated the relationship between offspring sex and egg mass, both in a within and a between clutch comparison in black-headed gulls, a species with similar life-history and sexual size dimorphism to the lesser black-backed gull.

In lesser black-backed as well as black-headed gulls, males are reported to be more sensitive to a reduction in egg size, egg quality and post-hatching food conditions than females (Griffiths, 1992; Nager *et al.*, 1999, 2000, W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished). Hence, sex ratio adjustment such as found under extreme experimental conditions with repeated egg removal (Nager *et al.*, 1999), is therefore very likely of relevance to compensate the enhanced male sensitivity. We therefore hypothesized that females transfer more resources to male oocytes or vice versa allocate the male sex to oocytes of higher quality.

We addressed the question whether variation in absolute egg mass is the decisive factor in between clutch sex ratio adjustment among gulls species. Alternatively, each individual female may vary egg mass and offspring sex within her clutch in terms of a maternal strategy of sex specific resource allocation.

Material and methods

Study species and study population

The Black-headed gull is a sexually size-dimorphic species with adult males about 15% heavier and skeletally larger than adult females (Glutz von Blotzheim & Bauer, 1982). Their clutch typically consists of three eggs, which are laid over a 3–6-day period (Cramp & Simmons, 1983). On average the eggs are incubated for 23 days, after which the chicks hatch asynchronously (Glutz von Blotzheim & Bauer, 1982). In 2000 and 2001, fieldwork was conducted in a large black-headed gull colony (about 2000 breeding pairs distributed over several sub-colonies) situated on the Northeast coast of The Netherlands.

Field work and data collection

During the laying period, nests were controlled every day for freshly laid eggs. Each egg was marked with a nontoxic pen for its position within the laying order (A for first, B for second and C for last laid eggs) and the date of egg laying. On the day of clutch completion, the nest was marked with a numbered bamboo stick, and all eggs were weighed to the nearest 0.1 g. As first and second eggs were weighed a few days post-laying, their mass might be slightly underestimated. In 2000, all clutches remained in the field, where hatching took place. In this year we determined the sex (see below) of all three chicks of a clutch in 292 nests. At the time of hatching, all nests were checked once a day for freshly hatched chicks. Whenever a chick started to hatch, with its beak showing through the broken eggshell, we marked the bill with a nontoxic pen, in order to register which chick came from which particular egg. At the day of hatching all chicks received a numbered colour ring for individual identification. From each hatchling we collected a drop of blood (about 20 μL) by puncture of the ulnar vein and stored it in 100% ethanol. Although we did not establish the hatchling mass in the course of this experiment, previous work on our species has shown that hatchling mass strongly correlates with egg mass (Eising *et al.*, 2001). In 2001, none of the 142 clutches was allowed to hatch under natural circumstances. Of these clutches, 96 were collected on the day of clutch completion and placed in an incubator at 37.5 °C with 60% humidity to allow some embryonic development for sex determination. As some incubation takes place prior to clutch completion, the eggs were incubated differentially to approximately equalize the total incubation time (60 h in case of the first laid egg, 72 h for the second-laid egg and 84 h in case of the last-laid egg). Embryo and yolk of these eggs were separated. The yolk was used for other purposes (Müller *et al.*, 2004) and a small tissue

sample of the embryo stored in 100% ethanol until analysis. Eggs of the remaining 46 clutches were taken to the incubator about 4–7 days before hatching. These eggs hatched in the incubator and within a few hours after hatching we sampled a small amount of blood (see above). These chicks were subsequently cross-fostered as part of another experiment (W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished).

Molecular sex identification

About 1 μL of the blood sample, and in case of the embryos a small tissue sample, was used for Chelex® resin-based DNA extraction (Walsh *et al.*, 1991). Two microlitres of the resulting DNA solution was used in a polymerase chain reaction (PCR) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (for details see Griffiths *et al.*, 1998). The amplified products were separated in 2.5% agarose gels containing ethidiumbromide and visualized under UV light. Based on the presence of the PCR-products, hatchlings were identified as male (CHD-Z gene product only) or female (CHD-Z gene and CHD-W gene products). The reliability of this method has been evaluated with Black-headed gulls of known sex (see Müller *et al.*, 2003), yielding a 100% correct match.

Statistical analyses

Sex ratio data were analysed using hierarchical linear modelling in the MLwiN program, version 1.10 (Rasbash *et al.*, 2000). This method takes into consideration the nested relationship of different chicks in a nest in order to prevent pseudo replication and controls for multiple (independent) variables. Sex ratio data were first transformed using the logit function and their order penalized by quasi-likelihood estimation (Goldstein, 1995). Significance was then tested using the Wald statistic, which follows a χ^2-distribution. As we were interested in within nest effects separated from the between nest variance, mean nest values of the tested variables were included in the model yielding separate estimates for within and between nest effects (Snijders & Bosker, 1999). The following independent variables were included in the first model: relative egg mass (egg mass/mean egg mass) (within clutch estimate), mean egg mass per clutch (between clutch estimate), position in the laying sequence (categorical variable), laying date, year and all possible interactions. *Post hoc* tests were in the same program. Changes in egg mass with laying sequence were analysed in the MLwiN program as described above. In this second model we included: A–C mass-asymmetry (egg mass A-egg – egg mass C-egg), position within the laying sequence (categorical variable), laying date, year and all possible interactions.

Egg mass in relation to laying position was analysed in MLwiN, here significance was tested using the increase in deviance (Δdeviance), when a factor was removed from the model, which follows a χ^2-distribution (Wald statistic). To analyse the distribution of the sexes over the laying sequence we used Binomial tests, in case of the analysis of changes in egg mass with laying interval (=difference in laying date between first and last laid egg) linear regressions.

There were no significant contributions of year ($P > 0.26$ in all cases) or laying date ($P > 0.59$ in all cases) and all their two-way interactions in none of the analyses. In the absence of specific predictions with respect to the effects of these factors on sex ratio, the detailed non-significant statistics regarding year and laying date are not repeatedly presented in the results section.

Results

Between nest comparison

The overall sex ratio in our sample of 1302 eggs/chicks from 434 nests was 49.8% males. Figure 1 shows the brood sex ratios as a function of average egg mass per clutch. Variation in average egg mass per clutch did not co-vary with the brood sex ratio (Wald $\chi^2_1 = 0.14$, ns, Fig. 1, Model 1, Table 1). Egg mass data, presented in Table 2, showed considerable variation, ranging from 27 to 47.2 g. However, there was also no significant

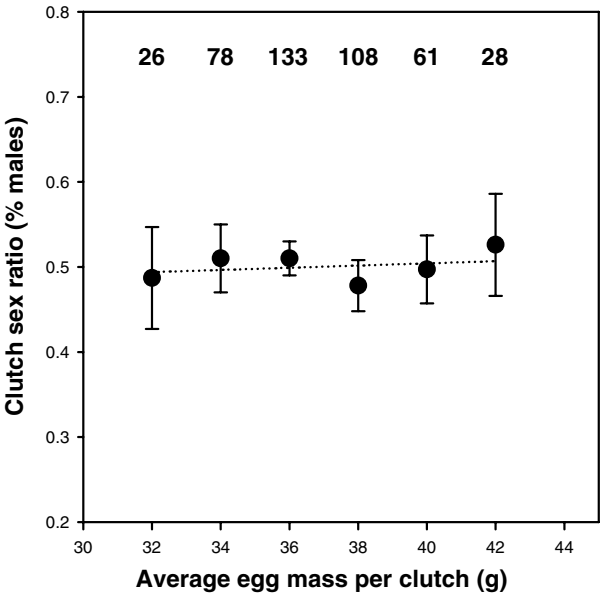

Fig. 1 Percentage of males in relation to the mean egg mass per clutch binned in average size classes of 2 g each. Data were pooled if the sample size per group dropped below the minimum of 10 cases which was the case at both ends of the distribution. The respective sample sizes are indicated by the numbers. Statistical tests were carried out on individual data points (see text), and revealed no significant relationship (dotted line).

Table 1 Offspring sex in relation to relative egg mass (within clutch variation) and average egg mass (between clutch variation) (Model 1) and A–C mass asymmetry, laying order, and the interaction between those parameters (Model 2).

	Explanatory terms	χ^2	d.f.	*P*-value
Model 1	Relative egg mass	8.51	1	<0.005
	Mean egg mass	0.14	1	ns
Model 2	A–C mass asymmetry	3.92	1	0.05
	Laying order	2.54	2	ns
	A–C mass asymmetry * laying order	10.01	2	<0.01

All parameters and interactions that did not contribute significantly to the model were excluded and significance levels for the remaining factors were adjusted accordingly, these values are presented.
d.f., Degrees of freedom; ns, not significant.

Table 2 Clutch mass and egg mass in relation to position in the laying sequence.

Variable	Mean (g)	SE	Min (g)	Max (g)	Range (g)
First-laid egg	37.11	0.14	28.8	46.8	18.0
Second-laid egg	37.34	0.14	29.9	47.2	17.3
Third-laid egg	36.12	0.14	28.0	45.8	17.8
Clutch mass	110.51	0.38	88.1	136.3	48.2

$n = 1302$ eggs out of 434 clutches.

relationship when A-eggs (Wald $\chi^2_1 = 1.89$, ns), B-eggs (Wald $\chi^2_1 = 0.76$, ns) or C-eggs (Wald $\chi^2_1 = 0.21$, ns) were analysed separately in *post hoc* tests.

Within nest comparison

Offspring sex was not significantly associated with the laying order and was thus not maintained in Model 1 (sex ratio of A-egg 0.52, B-egg 0.47, C-egg 0.50; Wald $\chi^2_2 = 2.21$, ns).

Within each clutch, males hatched from relatively heavier eggs compared to females (Wald $\chi^2_1 = 8.51$, $P < 0.005$, Model 1, Table 1) (Fig. 2). Similar trends occurred in all three laying positions (*post hoc*: A-egg: Wald $\chi^2_1 = 3.61$, $P = 0.06$; B-egg: Wald $\chi^2_1 = 1.83$, $P = 0.18$; C-egg: Wald $\chi^2_1 = 4.36$, $P < 0.05$) (Fig. 2).

We found that the second laid egg was more likely to be a male egg, if the first laid egg had been a male egg, which also applied for females. Thus the probability for two eggs of the same sex within the first two eggs of a clutch was significantly enhanced (58% same sex, Binomial test, $P < 0.001$). However, this did not apply for the first versus last (49% same sex, Binomial test, ns) and the second vs. last egg (51%, Binomial test, ns). This may relate to changes in egg mass, as first and second egg differed only slightly in weight (mean change +0.22 g ± 0.09 SE, Δdev_1 6.497, $P < 0.05$), in contrast to the strong change in egg mass between first and last laid egg (mean change −0.99 g ± 0.11 SE, Δdev_1 79.72, $P < 0.001$).

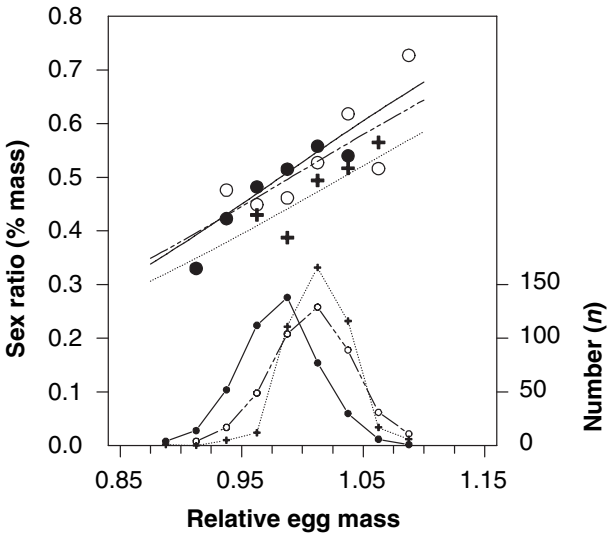

Fig. 2 Percentage of males (left *Y*-axis) in relation to the relative egg mass (individual egg mass divided by the clutch mean) binned in classes of 0.025, separately for each laying position. If the sample size per group dropped below the minimum of ten cases, which was the case at both ends of the distribution, these data were pooled. Statistical tests were carried out on individual data points (see text). Open circles represent first laid eggs (dashed-dotted regression line), crosses second laid eggs (dotted regression line), closed circles last laid eggs (solid regression line). Sample sizes for first, second and last-laid eggs (right *Y*-axis) are given as frequency distribution of relative egg masses, using identical symbols.

We further analysed the relationship between within-clutch changes in egg mass (A–C mass-asymmetry) and offspring sex, as egg mass may increase or decrease over the laying sequence. Clutches that either showed an increase or decrease in egg mass with laying sequence did not differ in their brood sex ratio (Wald $\chi^2_1 = 0.09$, ns). In contrast, they did differ in the distribution of the sexes over the laying sequence. In clutches with relatively heavy first eggs, male chicks were more likely to hatch from the first and females from the last egg. If the heaviest egg was laid at the end of the sequence the distribution of the sexes over the laying sequence followed the opposite pattern (Fig. 3, interaction term A–C mass-asymmetry * laying rank: Wald $\chi^2_2 = 10.01$, $P < 0.01$; *post hoc*: A-egg: Wald $\chi^2_1 = 3.76$, $P = 0.05$; C-egg: Wald $\chi^2_1 = 4.851$, d.f. = 1, $P < 0.05$, Model 2, Table 1). The A–C mass-asymmetry was not correlated with the total clutch mass (Pearson correlation $r = 0.02$, ns), but was related to the laying interval. With an increasing laying interval (duration between laying of the first and the last egg), egg mass of the last egg decreased significantly ($n = 421$, $t_1 = -2.76$, $P < 0.01$), in contrast with the first egg of the clutch ($n = 421$, $t_1 = 0.29$, ns) (Fig. 4). This caused a significant change in A–C mass-asymmetry with increasing laying interval (linear regression: $n = 421$, $t_1 = 4.03$, $P < 0.001$).

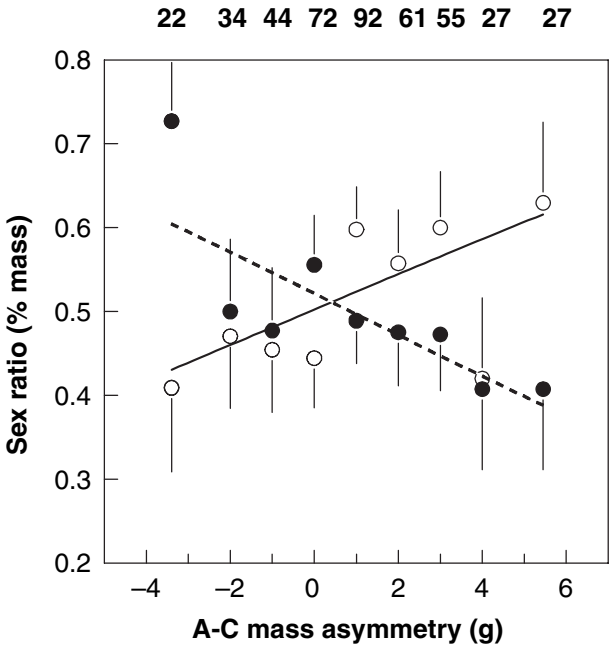

Fig. 3 Sex ratio of first- (open circles, solid regression line) and last-laid eggs (filled circles, dotted regression line) in relation to the A–C mass-asymmetry within the clutch [mass of first-laid egg (A-egg) minus mass of last-laid egg (C-egg)]. Sample sizes are indicated by the numbers and standard errors of the mean are indicated by bars. Data were pooled if the sample size per group dropped below the minimum of 10 cases. Statistical tests were carried out on individual data points (see text).

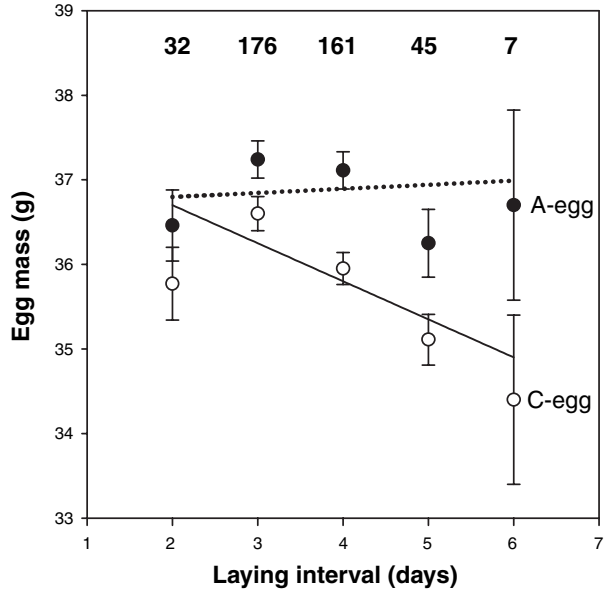

Fig. 4 Average egg mass (±SE) of first laid eggs (A-egg, filled circles, dotted regression line) and last laid eggs (C-egg, open circles, solid regression line) in relation to the laying interval, binned in 1 day categories.

Discussion

Based on the empirical evidence that a reduction in egg size and egg quality causes retarded growth in male black-headed gulls and reduces the survival probability in male but not female lesser black-backed gulls (W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished; Nager *et al.*, 1999), we expected light clutches to be biased towards females, the less costly and less vulnerable sex. However, we did not find any evidence that light clutches were female-biased (Fig. 1). Also Alonso-Alvarez & Velando (2003) did not find such a relationship in yellow legged gulls *Larus cachinnans*. At the individual level over a large range of egg masses (see Table 2), male eggs were not significantly heavier. This indicates that egg mass itself is not the decisive factor for adjustment of the embryonic sex in our study species.

Although egg mass determines how much nutrients are available for the developing embryo, because of their genetic background or their skeletal size, individual females are constrained in varying their egg size (reviewed by Williams, 1994; Christians, 2002). This hampers a comparison of an inter-relationship of offspring sex and egg mass between individuals. However, females may differ in their allocation pattern between subsequent clutches or between different eggs within their clutch. This underlines the importance of distinguishing between within clutch effects and between clutch effects when analysing the relationship between egg mass and offspring sex. When analysing the relationship between egg mass and embryonic sex in a within clutch context, we did find evidence that sex ratio adjustment occurred, with lighter eggs within each clutch bearing females and heavier eggs males (Fig. 2).

The lack of a relationship between absolute egg mass and embryo sex in the studies using an egg removal approach in other species (Nager *et al.*, 1999; Kalmbach *et al.*, 2001) may be caused by the fact that females were not allowed to complete a natural clutch. Hence, they could not adjust egg mass and offspring sex in interplay with the laying sequence, i.e. relative to the eggs already present in the nest, as each egg was removed immediately after laying. Furthermore, both studies did not differentiate explicitly between within and between clutch effects when analysing the association between egg mass and offspring sex. The importance of such an approach is clearly indicated by our results. Egg mass, varying within and between clutches was not related to sex, but separating the two sources of variation revealed a significant within clutch association between (relative) egg mass and sex of the egg.

The distinction between these two possibilities has clear consequences for our understanding of sex allocation. Our results suggest that females optimize the distribution of sexes within their clutch in relation to their individual context. The lack of consistency among

studies on sex allocation looking at single effects in a between female comparison might hence partly relate to individual integration of different effects by each female. An association between laying sequence and offspring sex for example strongly depends on the within clutch egg mass asymmetry as shown in this study. A similar context-dependency of sex ratio adjustment in interplay with laying order has also been shown by Badyaev *et al.* (2002).

Our results furthermore affect the mechanistic as well as functional explanations of the inter-relationship between egg mass and sex.

Black-headed gulls show extensive variation in egg mass within the clutch. The last laid egg is frequently the smallest in the sequence (modal clutch size three eggs). It may indicate the difficulties of females to maintain egg mass in the course of laying. Indeed, within clutch variation in egg mass of gulls has been shown to be influenced by the condition of the female and the environmental food situation. Females in enhanced condition increase the relative size of the last laid egg (e.g. glaucous-winged gull *Larus glaucescens*: Reid, 1987, lesser black-backed gull: Hiom *et al.*, 1991). In our study, this is supported by the fact that females with longer inter-egg intervals laid smaller final eggs (Fig. 4), but did not lay lighter clutches. The A–C mass-asymmetry may reflect the allocation pattern within the range of an individual female as an increase in egg size across the laying sequence has been shown to enhance the survival probability of later hatching chicks (herring gull *Larus argentatus*: Kilpi *et al.*, 1996, see also red winged blackbird *Aegelius phoeniceus* Blank & Nolan, 1983).

In clutches where first laid eggs were heavier than last laid eggs (positive A–C mass-asymmetry), male offspring was allocated to the first hatching position and a female to the last (Fig. 3). Given the higher sensitivity of male chicks towards egg size and quality (Nager *et al.*, 1999, W. Müller, C.M. Eising, T.G.G. Groothuis & C. Dijkstra, unpublished) and the higher vulnerability of the larger sex when hatching late (bald eagle *Haliaeetus leucocephalus*: Dzus *et al.*, 1996, blue-footed booby *Sula nebouxii*: Torres & Drummond, 1997, jackdaw: Arnold & Griffiths, 2003), this maternal strategy may increase the reproductive output.

In contrast, clutches with last laid eggs being heavier than first laid eggs (negative A–C mass-asymmetry) tended to hatch a male from the last and a female from the first egg. Again, hatching more male chicks from the heaviest eggs within the clutch may relate to a different sensitivity of the sexes towards egg size and quality, whereas the adaptive significance of the female bias in the first-laid egg may relate to the interference competition and the dominance of the larger sex (Bortolotti, 1986; Anderson *et al.*, 1993b; Velando *et al.*, 2002) and extra need of females for the competitive advantage of hatching first under these specific circumstances.

As first and second laid eggs tended to be very similar in mass (Fig. 2), the observed relationship between relative egg mass and sex of the egg would imply a high frequency of two chicks of the same sex among the first two eggs of the clutch. This is indeed what we found, as same sex duos were significantly overrepresented compared with mixed sex duos in the first and second laid eggs from the same clutch.

This implicates that the majority sex in a clutch is assigned to those eggs, which have the best prospects for survival.

In conclusion, both, clutches with positive A–C mass asymmetry (of females presumed to be in good condition) or clutches with negative A–C mass asymmetry (females of potentially poor condition) produced predominantly males in their heaviest egg and females in their smallest egg.

From a mechanistic point of view one could question whether females adjust the sex of the offspring in relation to egg mass or vice versa. Both mechanisms are possible. If yolk size determines final egg size, theoretically females could adjust the sex according to the size of the yolk, as the yolk is formed before meiosis takes place and the sex of the embryo is determined (Sturkie, 1986). Such a mechanism may be possible if the growth of different oocytes of an individual female is inter-dependent, allowing the female to 'identify' relative egg mass already when determining the sex of the first egg. Alternatively, since about two-thirds of the egg mass is albumen (reviewed by Williams, 1994) which is formed after sex determination, females may also increase the allocation of albumen in relation to the (randomly determined) sex of the offspring.

We cannot distinguish between these two possibilities. Nevertheless, we conclude that sex and mass of the eggs covary within black-headed gull clutches in an apparently adaptive manner, taking into account the higher vulnerability of males to reduced egg size/quality.

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